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Isolation and screening of biofilm forming *Vibrio* spp. from fish sample around south east region of Tamil Nadu and Puducherry

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ABSTRACT

Biofilm are group of microbial community enclosed inside a well-organized complex structure made up of extracellular polymeric substances. Biofilm forming bacteria are the growing concern of morbidity and mortality in aquatic population. Among the different biofilm forming bacteria, *Vibrio* spp. are pathogenic, diverse and abundant group of aquatic organism. Aquatic animals like fish, mollusks and shrimp harbors pathogenic and non-pathogenic forms of *Vibrio* spp. In the present study, *Vibrio* strains were isolated from various region of Tamil Nadu (Chennai, Cuddalore and Tiruchirappalli) and Puducherry. A total of 155 strains were isolated from various sampling sites, among them 84 (54.7%) were confirmed as *Vibrio* spp. through biochemical characterization and *Vibrio* specific 16S rRNA gene amplification. Among the 84 strains 50 strains were confirmed as biofilm formers and they were categorized as strong (10), moderate (17) and weak (23), based on their biofilm forming ability. Further the pathogenicity of all the *Vibrio* strains were tested through lytic enzyme production (protease, gelatinase and hemolysin) and swarming motility. After 16S rRNA gene sequencing, the 10 strong biofilm forming strains were *Vibrio alginolyticus* (TFM17, TFM30, and UFM24), *Vibrio parahaemolyticus* (TFM1), *Vibrio harveyi* (CUS2), *Vibrio* spp. (CUS4) and *Gammaproteobacteria* (TFM4, TFM19, TFM61, CUS5). The present study is a first report dealing with the biofilm forming *Vibrio* spp. isolation from south east coastal regions of Tamil Nadu and Puducherry. Further the informations in the present study will help in finding novel strategies to combat multi drug resistance biofilm formers in aquaculture industries.

1. Introduction

Globally, India is the second largest country in aquaculture industries, demonstrating through an incredible growth from 0.75 million tonnes in 1950–51–9.6 million tons in 2013-2014 (Handbook of Fisheries and Aquaculture, 2013, ICAR publication, India). India is having a large area of water bodies covering around 7517 km coastline, 195.210 km of an extensive river (14 major rivers) and canal system, 44 medium rivers, numerous small rivers and streams. Apart from this, 23.36 million ha is covering pond and tanks resources (Kumar, 2016). This is the major reason for the extensive development of skilled and accelerating aquaculture industries in the country. But the growing aquatic disease via bacterial pathogen is the major concern for the aquaculture sector causing serious threat to the economic and socio economic development of the country. In most cases, aquatic infections are majorly caused by the genus of *Vibrio* spp. which habitually thrives in marine environments. The temperature and other physicochemical condition of marine environment support the growth of *Vibrio* spp. Among the 30 genus of *Vibrio*, 14 species are pathogenic to human dwells in fish, shrimp and mollusks (Daniels et al., 2000). *Vibrio* spp. like *Vibrio cholera, Vibrio parahaemolyticus* and *Vibrio vulnificus* are mainly reported to cause sporadic infections, gastroenteritis, diarrhoea, abdominal cramps, nausea, vomiting, wound infections, septicaemia and sepsis (Daniels et al., 2000; Levine and Griffin, 1993).

The colonization of biofilm forming *Vibrio* over the host organism is supported by the flagella, pilli, exopolysaccharides, quorum sensing regulated factors and c-di – GMP signalling. Biofilm is the assemblage of microbial cells over any biotic and abiotic substrate (Yildiz and Visick, 2009). The biofilm matrix is composed of extracellular polymer substances (EPS) like exopolysaccharides, carbohydrates, proteins and eDNA. The initial adhesion of *Vibrio* spp. over the host cell is due to flagellum mediated motility (O'Toole et al., 2000). The biofilm matrix prevents the entry of foreign materials, antimicrobial compounds and the host immune system (Stewart, 1996; Donlan and Costerton, 2002).

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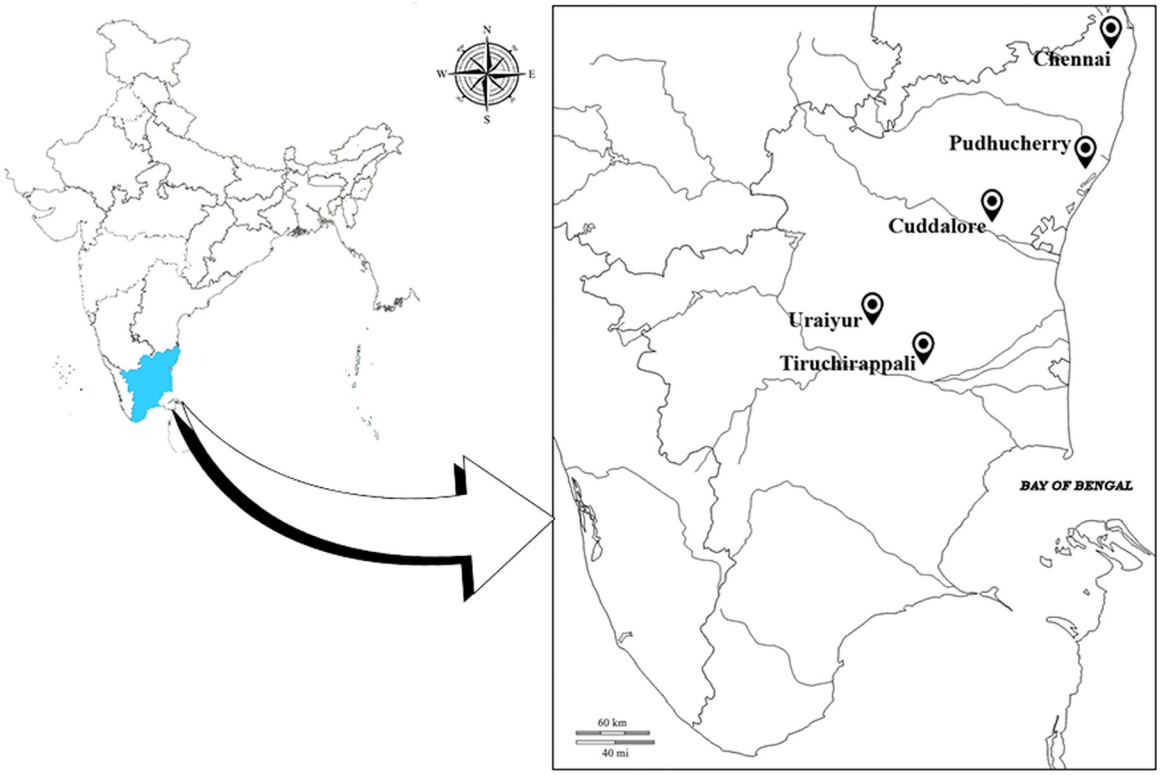

Fig. 1. Location of various sample collection sites from coastal and non coastal regions of Tamil Nadu and Puducherry.

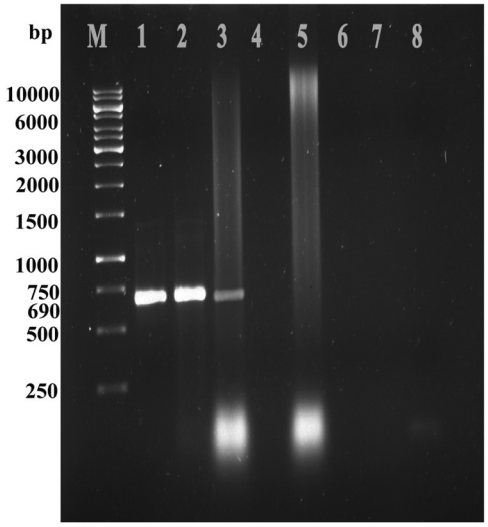

Fig. 2. Validation of *Vibrio* specific 16S rRNA Primer with various test organisms. Lane 1.*V. cholera* O1; Lane 2. *V. parahaemolyticus*; Lane 3. *V. alginolyticus*; Lane 4. *Serratia marcescens*; Lane 5. *Pseudomonas aeruginosa*; Lane 6. *Staphylococcus aureus*; Lane 7. *Staphylococcus epidermidis* and Lane 8. Negative control.

Frequent exposure or usage of antibiotics towards *Vibrio* infection and disease has led to the development of drug resistant strains and has improved the virulence of the bacteria (Defoirdt et al., 2011).

Generally, three types of pili namely mannose-sensitive haemagglutinin type IV pili (MSHA), toxin co-regulated pili (TCP) and chitin- regulated pili (ChiRP; formerly termed PilA) plays a crucial role in *Vibrio* spp. biofilm formation (Chiavelli et al., 2001; Meibom et al., 2004; Reguera and Kolter, 2005; Watnick et al., 1999). Apart from this, capsular polysaccharides (CPS) and *Vibrio* polysaccharides (VPS) loci are also involved in the biofilm formation (Yildiz and Schoolnik, 1999). Biofilm formation and colony morphology are correlated because cells in a colony are capable of producing rigid structural exopolymers forming rigid, rugose and mucoid colonies (McCarter, 1998; Enos-Berlage and McCarter, 2000). In addition, the transcriptional regulators for signal transduction and quorum sensing are also involved in biofilm formation of *Vibrio* spp. Quorum sensing (QS) is a inter and intracellular communication mechanism relies on the density of cell population and induced through cell signalling molecules (Martinelli et al., 2004). The QS organizes variety of physiological functions including motility, conjugation, competence, sporulation, virulence and biofilm formation. In addition, some bacteria sense and respond to multiple autoinducer signals, and in natural habitats this could allow them to differentiate between species within a consortium (Hammer and Bassler, 2003). Three kinds of QS system has been identified in the *V. harveyi,* each one is comprised of an autoinducer synthase and a two-component receptor (LuxM and LuxS) (Henke et al., 2004). LuxM and LuxS synthesizes; Autoinducer-1 (AI-1) and acyl homoserine lactone (AHL) used for intraspecies communication (Bassler et al., 1993; Cao and Meighen, 1989). These signalling mechanism supports biofilm formation and pathogenicity of *Vibrio* spp. Considering the occurrence of vibriosis in aquatic animals which directly influence the economy and productivity of sea food industry, the present work is focussed on surveying the biofilm forming *Vibrio* spp. available in the coastal and non-coastal regions of Tamil Nadu and Puducherry. In addition biochemical and molecular techniques like 16S rRNA amplification and sequencing has been performed for the identification of the biofilm forming *Vibrio* spp. Therefore this report will give a clear idea on the biofilm forming *Vibrio* species present in Tamil Nadu and Puducherry.

2. Materials and methods

2.1. Sample sites and collection

Sample collections were performed in the markets of coastal and non-coastal region of Tamil Nadu and Puducherry. The major sample collection areas of Tamil Nadu include Chennai (13°07'25.6"N 80°17'49.4"E),

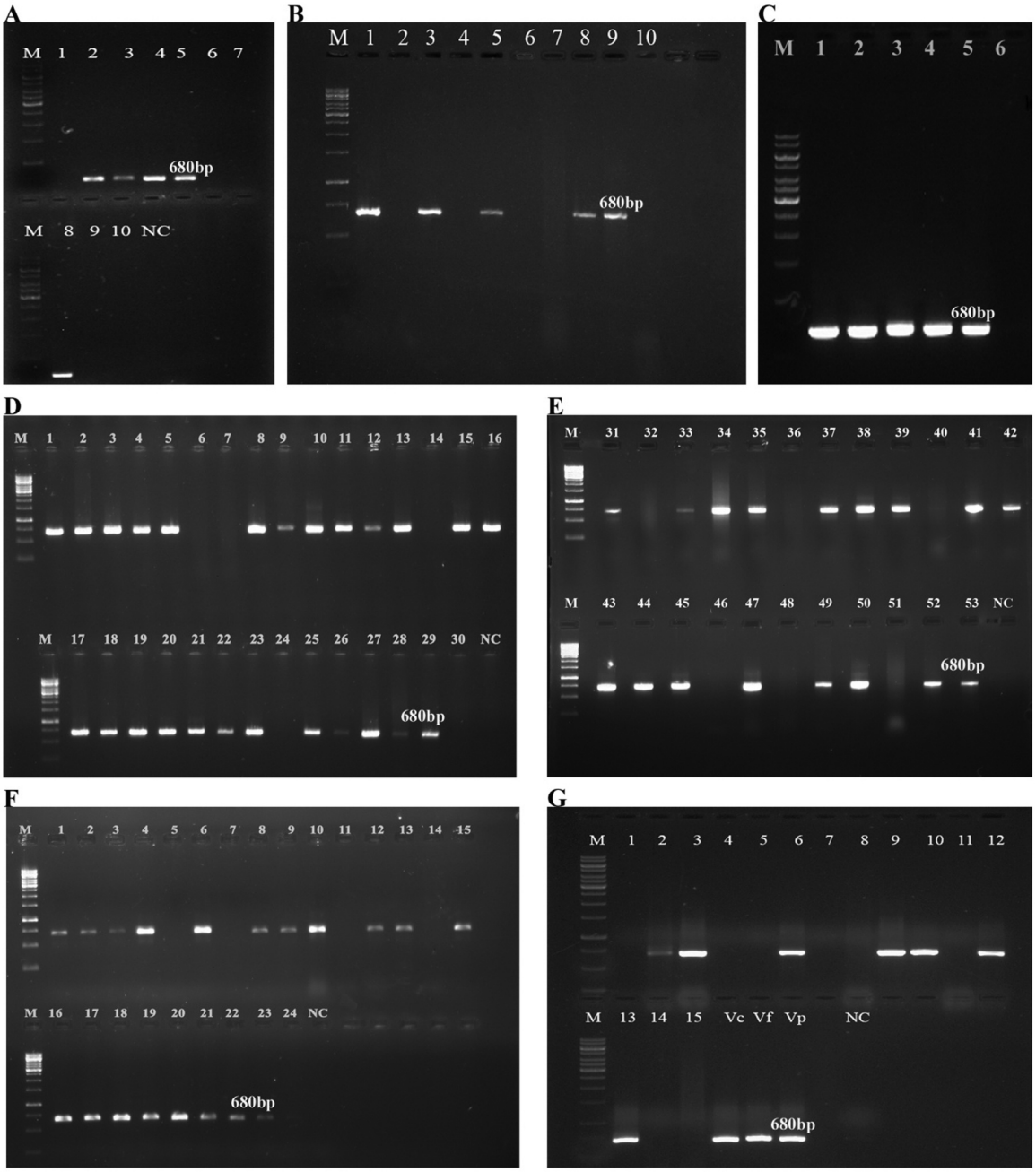

Fig. 3. Identification of *Vibrio* spp. from fish samples of Tamil Nadu and Puducherry through amplification of *Vibrio* specific 16S rRNA gene. A. Chennai beach sites (CHB; n = 5/10), (B) Chennai Kasimedu (CHK; n = 5/10), (C) Cuddalore Shrimp form (CUS; n = 5/5), (D&E) Tiruchirappalli Fish Market (TFM; n = 42/53), (F) Uraiyur Fish Market (UFM;n = 10/14) and Cuddalore, (G) Puducherry site (n = 7/15); Vc: *Vibrio cholera,* Vf: *Vibrio fluvialis*, Vp: *Vibrio parahaemolyticus*.

Cuddalore (11°42'58.3"N 79°46'28.6"E), Tiruchirappalli (10°48'56.1"N 78°41'57.2"E) and Puducherry (11°55'54.0"N 79°48'37.3"E). The samples were collected in aseptic plastic bag, which was tightly packed and transported to the laboratory in an aseptic condition.

2.2. Preparation of fish sample for the isolation of Vibrio spp

Fish samples collected from the sampling sites were chopped into fine pieces and 25 g of sample was inoculated in 225 ml of alkaline peptone water enriched with 3% NaCl (pH 8). The broth was incubated at 37 °C for 16 – 18 h. After incubation the pellicle of culture was taken and serial dilution was performed with 10 fold of saline water. From each dilution 100 µl was plated on the TCBS medium and incubated at 37 °C for 18–24 h (Kaysner and DaPaola, 2004).

2.3. Bacterial strains, culture condition and growth media

The reference bacterial strains of *Vibrio cholera* O1 was provided by Department of Biotechnology, Alagappa University, Karaikudi, India. Other *Vibrio* spp. like *Vibrio parahaemolyticus* ATCC17802, *Vibrio alginolyticus* ATCC17749 were procured from American Type Culture Collecction (ATCC). All the ATCC strains of *Vibrio* and isolated strains were grown in LB broth containing 1% NaCl and incubated at 37 °C. The cultures were also maintained in LB + 1% NaCl agar plate. Other bacterial reference strains like *Pseudomonas aeruginosa* PA14, *Serratia marcescens* (Genbank accession number FJ584421), *Staphylococcus aureus* ATCC11632 and *Staphylococcus epidermidis* ATCC12228 were grown in LB broth and agar at 30 °C for 24 h.

Table 1
Surveillance of biofilm forming *Vibrio* isolates from various fish samples of south east area of Tamilnadu, India.

Study sites	Tiruchirappalli	Uraiyur	Cuddalore		Chennai		Pudhucherry	Total
	TFM	UFM	CU	CUS	CHB	CHK	PY	
A	65	25	25	5	10	10	15	155
B	42	10	10	5	5	5	7	84
C	64.6%	40%	50%		50%		46.6%	59.5%

A – Number of isolates from each study area.
B – Number of isolates confirmed through *Vibrio* specific 16S rRNA gene amplification.
C – Percentage of *Vibrio* isolates in each sites.
TFM – Tiruchirapalli Fish Market, Tiruchirappalli, India.
UFM – Uraiyur Fish Market, Tiruchirappalli, India.
CU – Cuddalore, India.
CUS – Cuddalore- Shrimp Culture, India.
CHB – Chennai Marina Beach Fish Market, India.
CHK – Chennai Kasimedu Fish Market, India.
PY – Pudhucherry fish landing area.

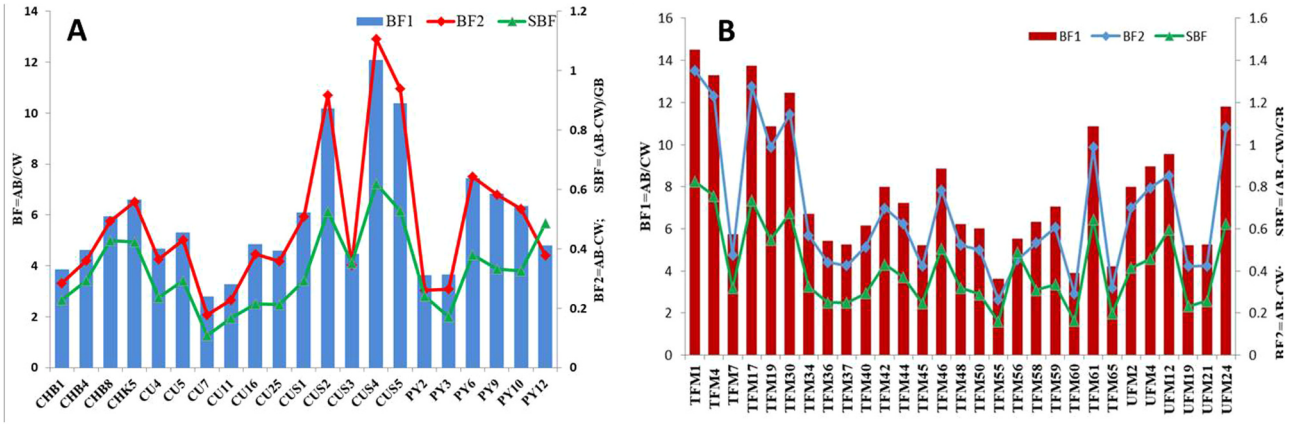

Fig. 4. A & B. Screening of biofilm forming *Vibrio* strains using quantitative analysis.

Table 2
Semiquantitative classification of biofilm Formation using three different formulas.

Formula	Strong	Moderate	Weak
BF 1 = AB /CW	≥ 10.0	≥ 6.0–9.9	≥ 3.0–5.90
BF 2 = AB - CW	≥ 0.900	≥ 0.50–0.89	≥ 0.20–0.49
SBF = (AB -CW) / G	≥ 0.50	≥ 0.4–0.4.9	≥ 0.2–0.39

BF – Biofilm formation.
AB – Stained attached bacteria; CW-stained control wells.
SBF – Specific biofilm formation.
G – Growth in suspended culture.
All values are OD595nm, Only G = OD600nm.

2.4. DNA extraction and amplification of Vibrio specific 16S rRNA gene

Vibrio strains were inoculated and grown in LB Broth containing 1% NaCl. The total genomic DNA was extracted using the procedure of Babu et al. (2009), and the extracted DNA was amplified with *Vibrio* specific 16S rRNA gene. The validation of *Vibrio* specific 16S rRNA gene primer was tested with other bacterial genomic DNA. The *Vibrio* specific reverse primer 680R - 5′-GAAATTCTACCCCCCTCTACAG-3′ and forward primer 5′- AGAGTTTGATCCTGGCTCAG - 3′was used for the study (Whitehouse et al., 2010). The polymerase chain reaction (PCR) was performed in 25 µl reaction mixture containing 50 ng of template DNA. The reaction was amplified in 35 cycles, each cycle consist of 94 °C for 30 s to, 55 °C for 30 s and 72 °C for 1.30 min (Applied Biosystem, USA). Approximately 680 bp amplified product was confirmed by running in 1% agarose gel with 1X TAE buffer.

2.5. Biofilm formation assay

The biofilm forming ability of the strains was determined using 24 well polystyrene plates. All *Vibrio* strains were grown overnight in 2 ml of LB broth + 1% NaCl medium at 35 °C. From the overnight incubation, 1% of bacterial suspension was inoculated in to 24 well plate containing 1 ml of LB containing + 1% NaCl in each well and incubated under static condition at 37 °C for 24 h. After incubation the broth containing free floating bacterial cells was quantified at 600 nm. The broth free wells were washed twice with 1 ml of sterile saline. The wells were stained with 1 ml of 0.4% crystal violet solution for 10 min and washed with sterile distilled water for the removal of excess dye. Later, the wells were destained with 10% glacial acetic acid for 30 min and the optical density was measured at 595 nm to quantity the biofilm formation in each well (Nithya and Pandian, 2010).

Quantification of the biofilm formed inside the well was determined using three different formulas (i) BF = AB – CW (Kadurugamuwa et al., 2003), (ii) BF = AB /CW (Soto et al., 2006) and (iii) SBF = (AB) CW)/G (Niu and Gilbert, 2004) where BF is the biofilm formed, AB is the bacterial cell stained with CV and CW is the control wells stained with CV; SBF is the Specific Biofilm Formation index and G is the density of which measured at 600 nm. All the assays were performed in triplicate.

2.6. Light microscopy

Morphological analysis of biofilm forming bacteria was performed using light microscope. The bacterial cells were allowed to form biofilm over a glass slide (2 × 2 cm) placed in the 24 well polystyrene plate. After incubation the glass slides were washed with saline and stained

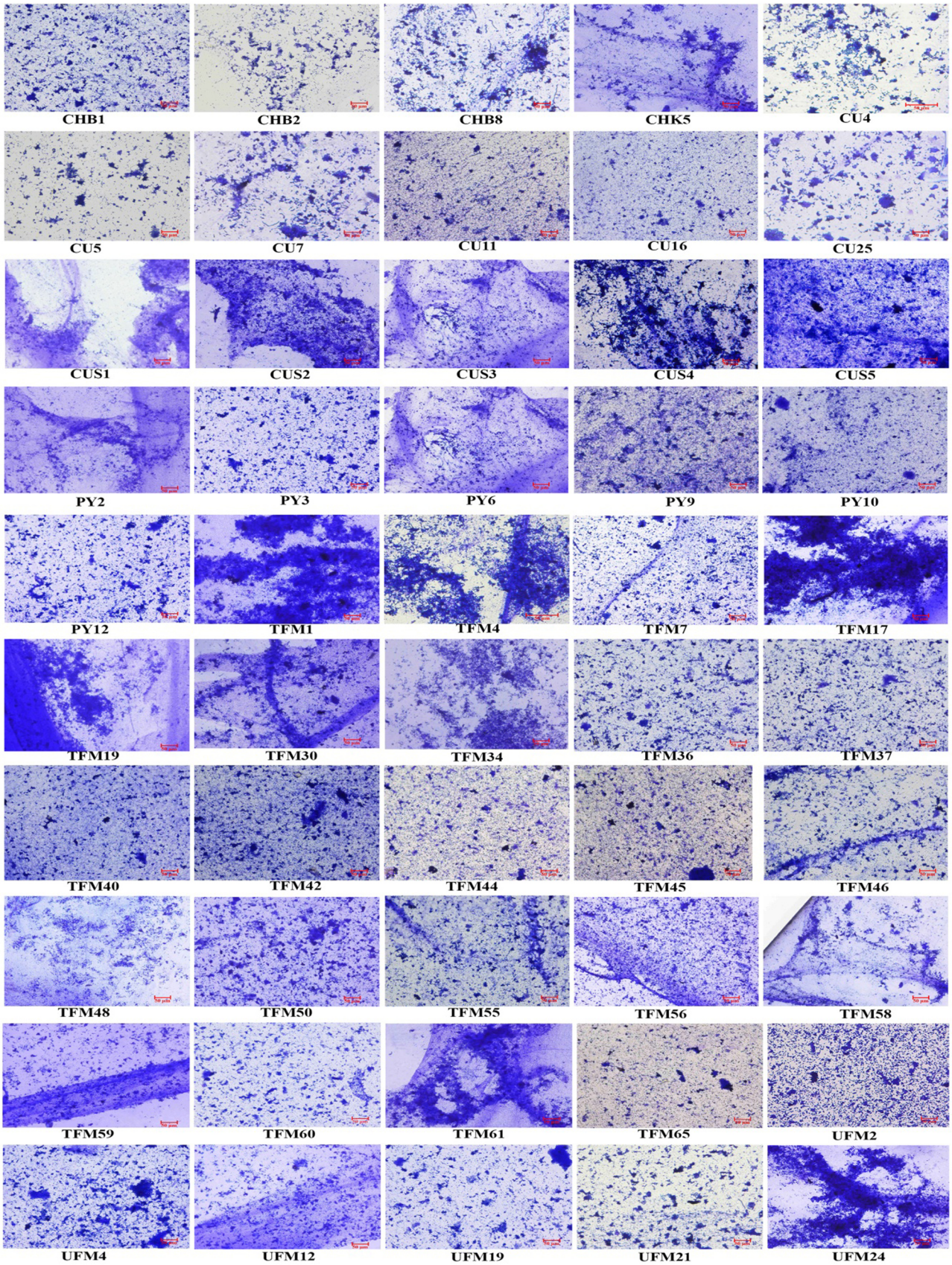

Fig. 5. Light microscopic observation for biofilm formation of *Vibrio* strains. The scale unit is 50 μm.

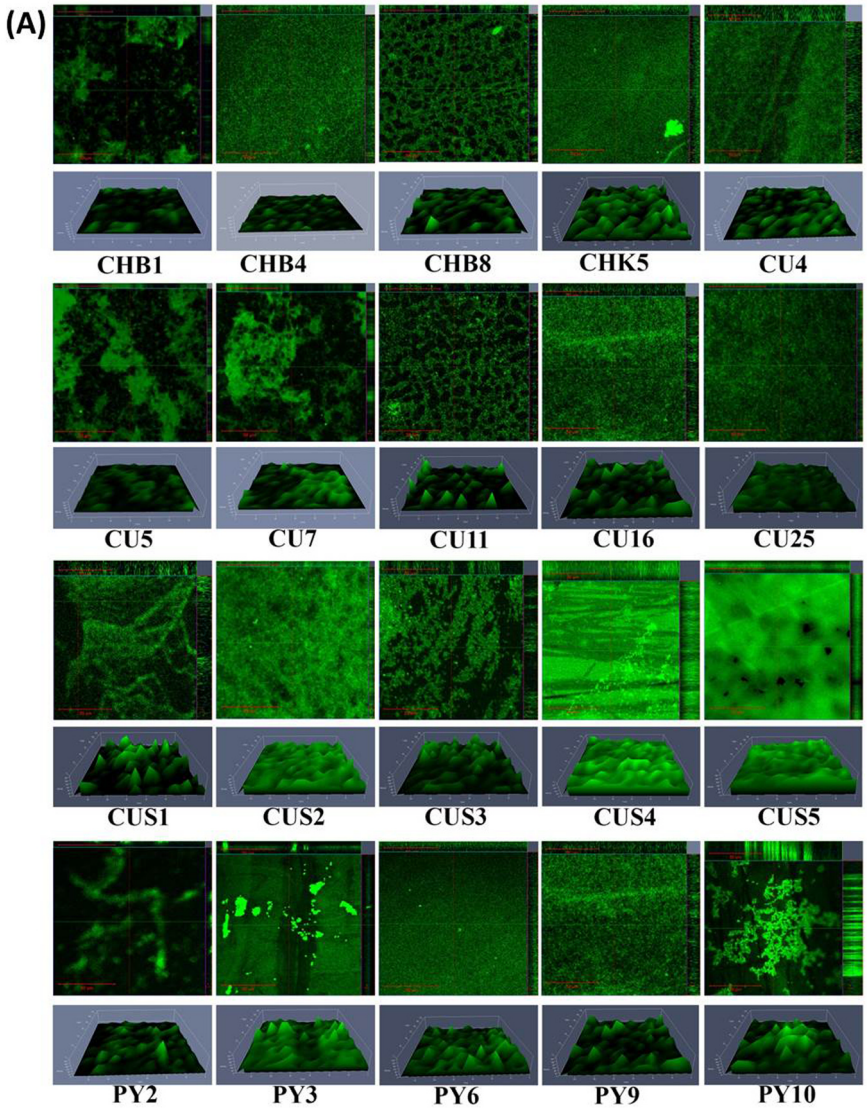

Fig. 6. A, B, C. Representative confocal laser scanning microscopy images of biofilms of *Vibrio* strains.

with crystal violet solutions as described previously. Then the excess dye was removed from glass slide by vigorous washing and observed under light microscope at 20X magnification and the images were documented (Nikon, Eclipse, Ti 100) (Chari et al., 2017).

2.7. Confocal laser scanning microscopy (CLSM)

The structural and surface topographic analysis of the biofilm attached over the glass slide was performed using confocal laser scanning microscope (CLSM). The glass slides inoculated with bacterial culture was stained with 0.1% acridine orange after 24 h of incubation as described previously. The biofilm were documented using CLSM at the excited range of 488 nm and fluorescence emission range was detected at 500–640 nm (Zen 2011 software; CarlZeiss) (Nithya and Pandian, 2010).

2.8. Swarming motility assay

The swarming motility of *Vibrio* strains was determined on swarming agar plate (1% peptone, 0.5% NaCl, 0.5% of glucose and 0.5% agar). 5 µl of *Vibrio* strains were spotted on center point of agar plate and incubated. After 24 h of incubation, the plates were observed for the formation of circular turbid zone from the center point of bacterial inoculums (Eberl et al., 1999).

2.9. Skim milk (protease) assay

Determination of protease was performed by streaking *Vibrio* spp. over the nutrient agar plates containing 1.5% of skim milk. After incubation of 24 h the plates were visualized for the appearance of clear zone around the colonies (Syngkon et al., 2010).

2.10. Gelatinase assay

Gelatinase activity was performed by nutrient agar supplemented with 0.4% gelatin. Overnight cultures of *Vibrio* spp. strains (10 µl) were added in wells (10 mm in diameter) made on gelatin agar plate. After 2 – 4 days of incubation, the plates were flooded with 10% HCl-15% $HgCl_2$ solution. The inhibition of gelatinase was determined by the appearance of clear zone assessment on the plates (Natrah et al., 2011).

2.11. Hemolytic assay

Hemolytic activity was performed by inoculating the *Vibrio* spp. on blood agar plates. The plates were incubated at 37 °C for 24 h. After 24 h of incubation, color change of the medium from red to green or a clear zone formation around the colonies confirmed the presence of hemolytic activity (Natrah et al., 2011).

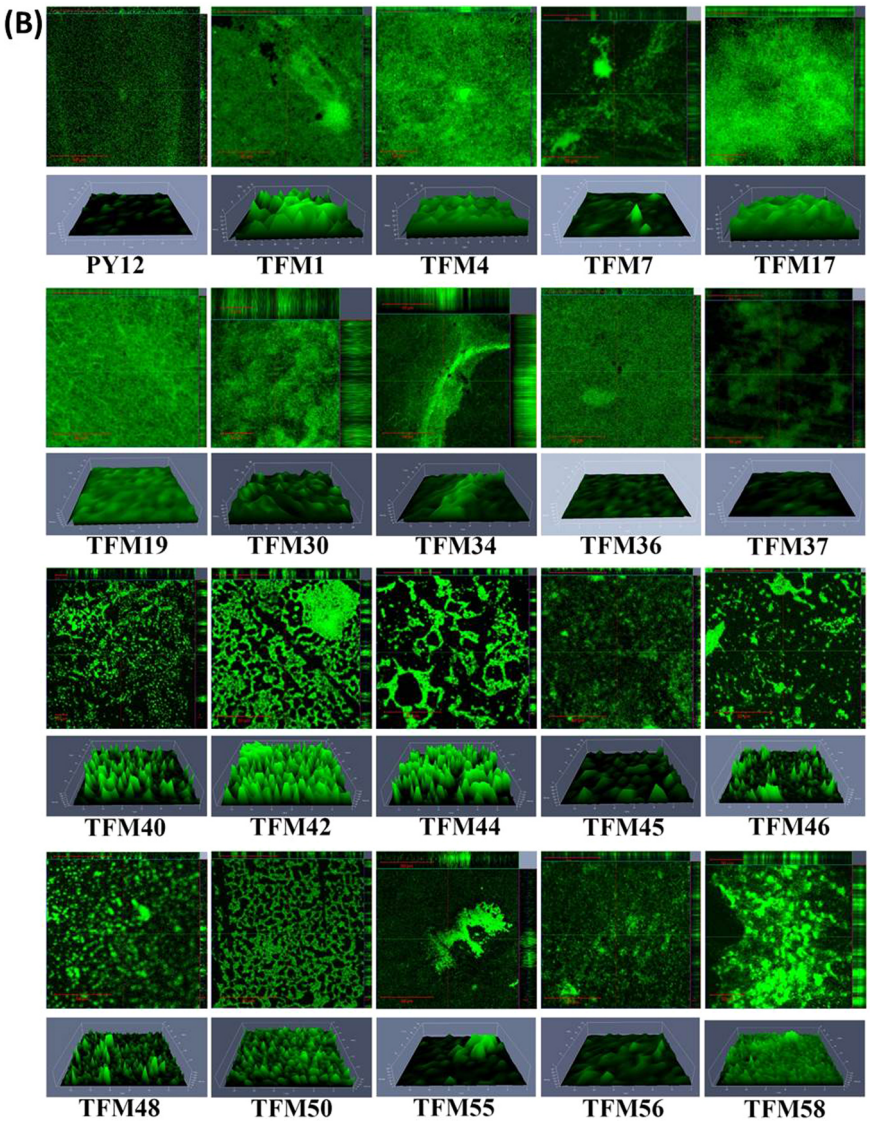

Fig. 6. (*continued*)

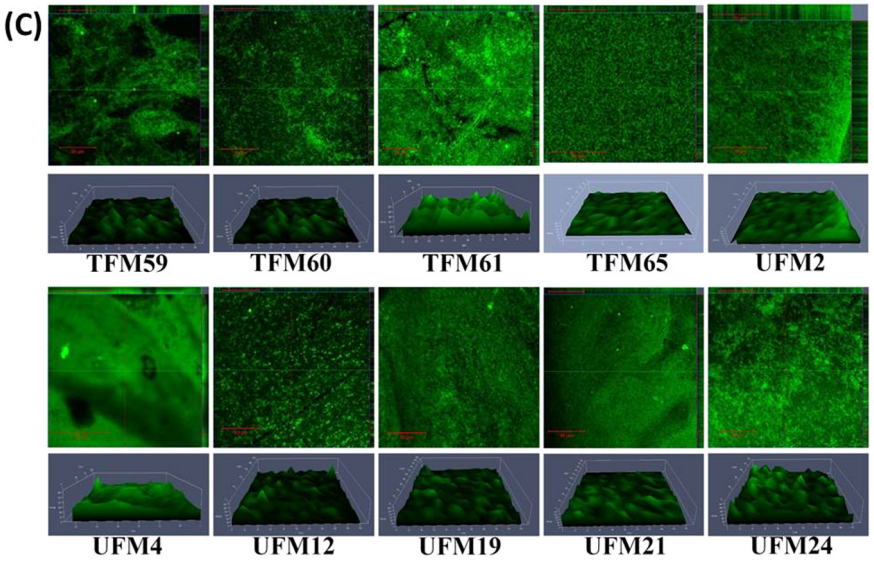

Fig. 6. (*continued*)

Table 3
Characterization of various quorum sensing mediated pathogenicity in *Vibrio* isolates.

S.no.	Vibrio isolates	Biofilm formation	Swarming	Protease	Gelatinase	Heamolysin
1	CHB1	W	+	+	+	+
2	CHB4	W	+	+	+	+
3	CHB8	W	+	+	+	+
4	CHK5	M	+	+	+	+
5	CU4	W	+	+	+	+
6	CU5	W	+	+	+	+
7	CU7	W	+	+	+	+
8	CU11	W	+	+	+	+
9	CU16	W	+	+	+	+
10	CU25	W	+	+	+	+
11	CUS1	M	+	+	+	+
12	CUS2	S	+	+	+	+
13	CUS3	W	+	+	+	+
14	CUS4	S	+	+	+	+
15	CUS5	S	+	+	+	+
16	PY2	W	+	+	+	+
17	PY3	W	+	+	+	+
18	PY6	M	+	+	+	+
19	PY9	M	+	+	+	+
20	PY10	M	+	+	+	+
21	PY12	W	+	+	+	+
22	TFM1	S	+	+	+	+
23	TFM4	S	+	+	+	+
24	TFM7	W	+	+	+	+
25	TFM17	S	+	+	+	+
26	TFM19	S	+	+	+	+
27	TFM30	S	+	+	+	+
28	TFM34	M	+	+	+	+
29	TFM36	W	+	+	+	+
30	TFM37	W	+	+	+	+
31	TFM40	M	+	+	+	+
32	TFM42	M	+	+	+	+
33	TFM44	M	+	+	+	+
34	TFM45	W	+	+	+	+
35	TFM46	M	+	+	+	+
36	TFM48	M	+	+	+	+
37	TFM50	M	+	+	+	+
38	TFM55	W	+	+	+	+
39	TFM56	W	+	+	+	+
40	TFM58	M	+	+	+	+
41	TFM59	M	+	+	+	+
42	TFM60	W	+	+	+	+
43	TFM61	S	+	+	+	+
44	TFM65	W	+	+	+	+
45	UFM2	M	+	+	+	+
46	UFM4	M	+	+	+	+
47	UFM12	M	+	+	+	+
48	UFM19	W	+	+	+	+
49	UFM21	W	+	+	+	+
50	UFM24	S	+	+	+	+

S- Strong; M- Moderate; W- Weak.
(+) – Positive.
The Isolates name code abbreviations were previously mentioned in Table 1.

2.12. Antibacterial susceptibility test

The antibiotic susceptibility of all the biofilm formers was assessed through disc diffusion method. The following antibiotic discs were used: Ceftazidime/Clavulanic acid (30 mcg/disc), Ciprofloxacin (30 mcg/disc), Co-Trimoxazole (25 mcg/disc) and Tetracycline (30 mcg/disc). Sensitivity to antibiotics was determined after incubation for 24 – 48 h using MHA (Mueller-Hinton agar) (Chakraborty et al., 2001).

2.13. Identification of Vibrio species by 16S rRNA gene sequence analysis

The amplified products of all the strong biofilm forming *Vibrio* spp. strains (680 bp) were carried out in Macrogen (Seoul, Korea). The sequences were compared with NCBI data base using Basic Local Alignment Tool (BLAST). The sequences were aligned and phylogenetic tree was constructed with Mega 6.0.

3. Results and discussion

The present study deals with the isolation and characterization of biofilm forming *Vibrio* spp. from various fish samples (Red Snapper, India Treadfin Bream and Penaeus) from local fish markets in Tamil Nadu and Puducherry India. In the preliminary screening a total of 155 strains were isolated from various fish samples collected from Tamil Nadu (Trichy fish market, Uraiyur fish Market, Cuddalore shrimp farm, Chennai marina beach, Kasimedu fish market) and Puducherry (Fig. 1). The strains were selected based on their colony morphology like size, color, texture, margin, and surface elevation in the TCBS plates (Alsina and Blanch, 1994). The strains were further assessed by PCR amplification using *Vibrio* specific 16S rRNA gene primers. For validation of the *Vibrio* specific primers, PCR was performed in comparison with three different *Vibrio* strains (*Vibrio cholera* O1, *Vibrio parahaemolyticus* ATCC17802, *Vibrio alginolyticus* ATCC17749) along with some Gram

Table 4
Antibiotic sensitivity test of biofilm forming *Vibrio* strains.

S.no.	Vibrio isolates	CAZ	CF	COT	TET
1	CHB1	S	S	S	S
2	CHB4	S	S	S	T
3	CHB8	S	S	S	S
4	CHK5	R	S	S	R
5	CU4	S	S	S	S
6	CU5	S	S	S	S
7	CU7	R	S	R	R
8	CU11	R	R	R	R
9	CU16	S	S	R	R
10	CU25	R	S	R	R
11	CUS1	S	S	S	S
12	CUS2	S	S	S	S
13	CUS3	R	R	R	R
14	CUS4	S	S	S	S
15	CUS5	R	R	R	R
16	PY2	S	S	S	R
17	PY3	S	S	R	R
18	PY6	R	S	R	R
19	PY9	S	R	R	R
20	PY10	S	S	R	R
21	PY12	S	R	R	R
22	TFM1	R	S	R	R
23	TFM4	R	S	R	R
24	TFM7	S	S	S	R
25	TFM17	R	R	R	R
26	TFM19	S	S	R	R
27	TFM30	S	R	R	R
28	TFM34	S	S	S	R
29	TFM36	S	S	R	R
30	TFM37	S	S	S	S
31	TFM40	S	S	S	S
32	TFM42	S	S	R	R
33	TFM44	S	S	R	R
34	TFM45	R	S	R	R
35	TFM46	S	S	S	R
36	TFM48	R	S	R	R
37	TFM50	S	S	R	R
38	TFM55	S	S	R	R
39	TFM56	S	S	S	R
40	TFM58	S	S	R	R
41	TFM59	S	S	R	R
42	TFM60	S	S	R	R
43	TFM61	R	S	S	R
44	TFM65	R	S	R	R
45	UFM2	S	S	S	S
46	UFM4	S	S	R	R
47	UFM12	R	S	R	R
48	UFM19	S	S	S	S
49	UFM21	S	S	R	R
50	UFM24	S	R	R	R

CAZ - Ceftazidime/Clavulanic acid (30 mcg/disc).
CF - Ciprofloxacin (30 mcg/disc).
COT - Co-Trimoxazole (25 mcg/disc).
TET - Tetracycline (30 mcg/disc).
S – Sensitive to antibiotic.
R – Resistance to antibiotic.

negative and Gram positive bacteria like *Pseudomonas aeruginosa* PA14, *Serratia marcescens* [Genbank accession number FJ584421], *Staphylococcus aureus* ATCC11632 and *Staphylcoccus epidermidis* ATCC12228. After agarose gel electrophoresis, the amplification was observed only in *Vibrio* spp. and was absent in all other reference strains of Gram negative and Gram positive bacteria (Fig. 2). The result of the validation PCR confirms the specificity of the primer towards *Vibrio* spp. Out of 155 strains, 84 strains were confirmed as a *Vibrio* spp. (54.7%) by the presence of amplicon with approximate size of 680 bp (Fig. 3). The PCR result confirmed abundance of 64.6% (n = 42) of *Vibrio* spp. in Tiruchirappalli; 40% (n = 10) of *Vibrio* spp. in Uraiyur; 50% (n = 15) of *Vibrio* spp. in Cuddalore; 50% (n = 10) of *Vibrio* spp. in Chennai; 46.6% (n = 7) of *Vibrio* spp. in Puducherry (Table 1).

The quantification of biofilm formation showed around 59.5% (50 strains) of *Vibrio* strains are biofilm formers. Further, based on the biofilm quantification, the strains were characterized as strong, moderate and weak biofilm formers (Fig. 4**A & B**) **(**Table 2**)**. According to the author's knowledge, this is the first study in Tamil Nadu and Puducherry reporting the isolation and identification of biofilm forming *Vibrio* strains from fish samples. Among the total biofilm forming *Vibrio* spp. (50 strains), 10 strains were strong biofilm former, 17 strains were moderate biofilm former and 23 strains were weak biofilm formers. The pathogenicity of a biofilm forming bacteria depends on the adhesion, entry, establishment, multiplication and production of chemotaxins. The various steps involved from the adhesion till colonization of a pathogen on the host surface largely depends on the biofilm forming ability (Donnenberg, 2000). Hence the presence of strong biofilm formers in the isolated *Vibrio* strains reveals the pathogenicity and the threat of food born infections through these seafoods.

Further the qualitative analysis of the biofilm using light microscope and CLSM resolves the thickness, architecture and area coverage. Fig. 5 showed the light microscopic images of biofilm matrix formed by the *Vibrio* spp. The light microscopic images clearly demonstrates that the strong biofilm formers have high intense cluster of bacterial cells and the moderate and weak biofilm formers exhibited low intense of bacterial cell (Fig. 5). In addition, CLSM results confirmed the difference in the biofilm thickness among various strains of *Vibrio* spp. through 2D, 2.5D and 3D visualizations (Fig. 6**A, B, C**).

The ability to produce various quorum sensing mediated pathogenic factors like swarming motility, proteases, gelatinases and heamolysin production was tested for all the strains of *Vibrio* spp. (Table 3). The analysis showed that, the strains were able to exhibit swarming motility and lytic enzyme production. Haemolysin and protease are lytic enzymes directly linked to cause infection against both fish and shrimp (Liu et al., 1996; Sun et al., 2007). In *Vibrio* spp. the biofilm formation is a quorum sensing mediated pathogenicity, which relies on flagella, pili and exopolysaccharides production (Yildiz and Visick, 2009). Studies have reported that *V. alginolyticus* produces Autoinducer-2 (AI-2) to induce the quorum sensing system for activating the virulence factors like hemolytic activity, extracellular polysaccharides, protease and siderophores production (Rui et al., 2008; Wang et al., 2007; Ye et al., 2008).

Table 5
16S rRNA sequence analysis and accession numbers of *Vibrio* isolates were various fish samples of south east area of Tamil Nadu, India.

S.no	Isolates and accession number	Sequence size (bp)	Nearest type of strain	Sequence identity
1	CUS2 -MK007472	624	*Vibrio harveyi* - KT986106	100%
2	CUS4 -MK007474	618	*Vibrio* spp. – MG593728	100%
3	CUS5 -MK007991	611	*Gammaproteobacteria* – KY053159	99%
4	TFM1- MH990262	659	*Vibrio parahaemolyticus* – MH536241	97%
5	TFM4 -MH991704	636	*Gammaproteobacteria* – KY053159	99%
6	TFM17 - MK007325	618	*Vibiro alginolyticus* – MH368393	99%
7	TFM19 - MK007992	611	*Gammaproteobacteria* – KY053159	99%
8	TFM30 - MK007345	618	*Vibrio alginolyticus* – KR347340	100%
9	TFM61 - MK007993	611	*Gammaproteobacteria* – KY053159	99%
10	UFM24- MK007470	623	*Vibrio alginolyticus* – KU845389	100%

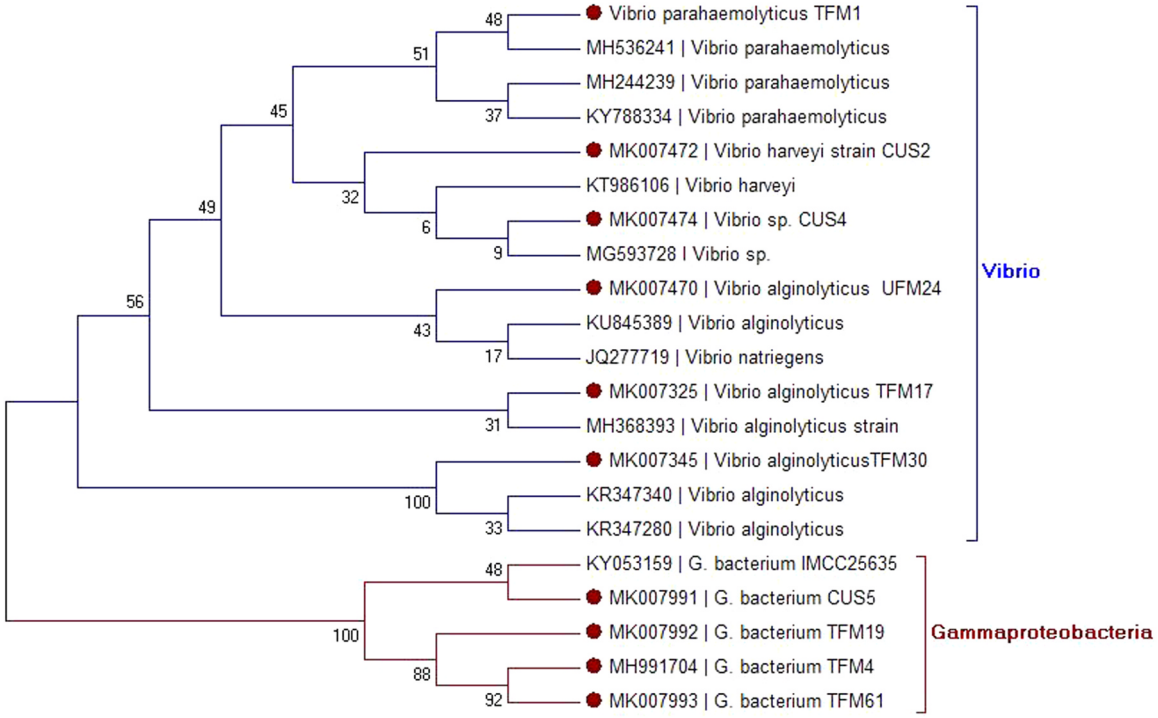

Fig. 7. Neighbour-joining phylogenetic tree from analysis of 16S rRNA gene sequence of various *Vibrio* strains from Tamil Nadu and Puducherry, India. The numbers at the nodes indicate the levels of bootstrap support based on 1000 resampled data sets. The scale bar represents 0.02 substitutions per nucleotide position.

The biofilm forming 50 strains were further screened for antibiotic susceptibility assay (Table 4). Among the biofilm formers few moderate and strong biofilm formers were competent to Ceftazidime/Clavulanic acid, Ciprofloxacin, Co-Trimoxazole and Tetracycline antibiotics. The antibiotic susceptibility test explored that the strong biofilm formers exhibited an elevated resistance towards antibiotics. These results further confirm that the biofilms prevent the entry of antibiotics and act as a shield to the residing bacteria (Costerton et al., 2003).

Among the 50 strains of *Vibrio* spp., the strong biofilm formers like CUS2, CUS4, CUS5, TFM1, TFM4, TFM17, TFM19, TFM30, TFM61 and UFM24 were taken for 16S rRNA gene sequencing. The sequence results were submitted on the NCBI Gene Bank and their accession numbers are given in Table 5. The sequences of the 10 strong biofilm forming *Vibrio* strains were analyzed through BLAST homology server. The strain TFM1 showed 97% similarity with *V. parahaemolyticus*. The strains TFM 17, TFM30, and UFM24 exhibited 99%, 100% and 100% similarity with *V. alginolyticus* respectively. TFM4, TFM19, TFM 61 and CUS5 were closely related to *Gammaproteobacteria* with 99% similarity, *Gammaproteobacteria* is a class of *Vibrionaceae* family. CUS2 strains showed similarity with *V. harveyi* and CUS4 was related with *Vibrio* spp. displayed 100% similarity (Fig. 7).

In India, *V. alginolyticus* has been reported to cause shell disease and white spot in *P. monodon*, shrimp and necrosis in *Macrobrachium rosenbergii* larvae (Lee et al., 1996). *V. alginolyticus* has been noted as major problem of antibiotic resistance in wound infections and ear soft tissue of human (Horii et al., 2005). *V. parahaemolyticus* is a well-known recognized pathogen of invertebrates including shrimp, abalone and *Haliotisdiversicolar supertexta* (Cai et al., 2007). In India the organism has been reported as major cause of red disease in *P. monodon* (Jayasree et al., 2006). Quorum sensing system contributed via LuxR homolg and OpaR in *V. parahaemolyticus* positively regulates opacity, cps gene and biofilm formation (McCarter, 1998). The secondary messenger is C-di-GMP controls the motility of bacterial strains and promotes biofilm formation. The increased level of cellular C-di-GMP in *V. parahaemolyticus* prevents the swarming motility and promotes the biofilm formation. In humans, *V. parahaemolyticus* cause acute gastroenteritis (Cho et al., 2008) with diarrhoea (DePaola et al., 2003; Drake et al., 2007). The bacterium produces thermostable direct hemolysin (TDH) and TDH related hemolysin (TRH) which is highly associated with human diseases (Miyamoto et al., 1969). Hence the presence of *V. parahaemolyticus* with swarming motility and hemolytic activity clearly depicts the pathogenicity and lethality of the strains.

In *Vibrio* spp., the flagella mediated motility was primarily involved in biofilm formation (O'Toole et al., 2000). As well as the lateral flagella switch to form swarmer cells which helps to move on surface and the highly viscosity of medium (Verstraeten et al., 2008). The swarming bacteria are capable to attain specific places and readily colonizes over the host for biofilm formation (Rather, 2005). Therefore the motility of swarming cells is proficient to form biofilm and consequently enhances the pathogenicity of a particular bacterial strain. This study is the first report on understanding the population of biofilm forming *Vibrio* spp. in the coastal and non-coastal region of Tamil Nadu and Puducherry. Overall the study clearly reveals the antibiotic resistance and pathogenicity of *Vibrio* via biofilm and Quorum sensing mechanism, which is a major cause for various infections in aquatic organism.

4. Conclusion

In precise the current study concludes that numerous strains of *Vibrio* spp. were isolated and identified from different regions of Tamil Nadu and Puducherry. The presence of biofilm formation, swarming motility and few lytic enzymes production authenticates the pathogenicity of the *Vibrio* strains. The biofilm forming ability has increased the susceptibility of *Vibrio* towards different antibiotics. These biofilm forming *Vibrio* strains causes potential and stable damage to the aquaculture industries and subsequently affects the human health. The incidence of the biofilm forming *Vibrio* spp. around the coastal and non-coastal area substantiates the need of novel strategies to combat the multi drug resistance biofilm formers in aquaculture industry. Ignoring the biofilm forming *Vibrio* will cause severe morbidity and mortality in aquaculture industries that directly affects the human beings and the country's economy. Therefore the need of the hour is to discover novel

strategies to target the biofilm formers, which will also conquer the antibiotic resistance. Thus our future aspects are mainly focussed on searching potential methods or ways to explore novel anti-pathogenic compounds from various resources.

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